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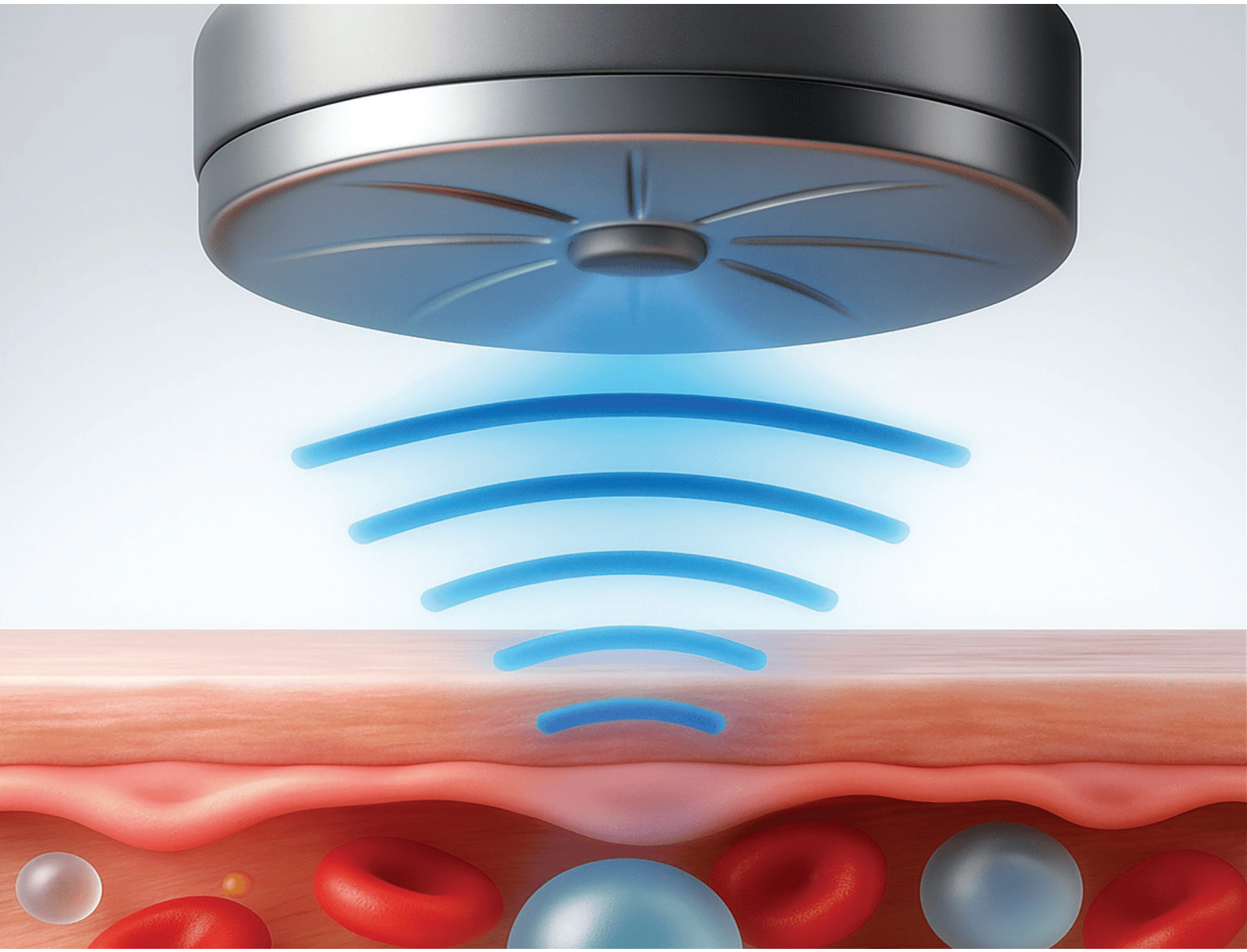

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Preliminary evidence for the combined efficacy of focused ultrasound blood–brain barrier opening and Re-1 delivery for anxiety and memory improvement in a 3xTg-Alzheimer's disease mouse model

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The aim of this preliminary study is to evaluate the efficacy of early intervention with Focused Ultrasound-induced Blood–Brain Barrier Opening (FUS-BBBO) and Re-1 delivery for anxiety amelioration, memory improvement, and pathology reduction in an Alzheimer's Disease (AD) mouse model. FUS-BBBO was applied and Re-1 delivered to the hippocampi of presymptomatic, male triple transgenic (3xTg)-AD mice using a preventative paradigm of 10 total biweekly treatments over the course of 5 months. Following treatment, the animals underwent five days of behavioral testing for anxiety, spatial memory, and reversal learning. The combination of FUS-BBBO and Re-1 delivery showed evidence of improving the long-term spatial memory and short-term reversal learning with no significant effect on amyloid and tau accumulation. The small sample size is a limiting factor for this preliminary study, which still offers promising indications in support of early intervention with amyloid-targeting Re-1 and FUS-BBBO for cognitive and minor pathological improvement in AD.

Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disease characterized by the accumulation of amyloid-ß plaques and hyperphosphorylated tau, in addition to cognitive decline.[1,2] AD is also associated with several neuropsychiatric symptoms, especially anxiety, with a prominence of 40%.[3,4] Additionally, AD patients exhibit memory and cognitive impairment with disease progression.[2] Aiming to mimic the behavioral and pathophysiological features of the disease, and to assess novel therapies, various animal models and behavioral tests have been established and standardized. The Open Field Test (OFT) is a robust behavioral paradigm for evaluating murine locomotion and overall anxiety.[5,6] The 3xTg-AD mouse model recapitulates both of these cognitive deficits, in addition to the pathological accumulation of Aß and tau.[7–9] The Active Place Avoidance (APA) paradigm has been described as an effective test for discerning hippocampal spatial memory and reversal learning through the training and reversal paradigms respectively.[10–12]

The results in some of these assays have strongly supported the fact that among the most recent and of high expectations therapeutic strategies against AD is the administration of monoclonal antibodies (mAbs), such as Lecanemab (Leqembi), which has been attempted to slow the accumulation of Aß plaques and cognitive decline in AD.[13] Monoclonal antibodies designed to bind amyloid plaques with high specificity mark these plaques for degradation and clearance by microglial or complement activation.[14] The high binding specificity of these mAbs yields efficient amyloid clearance, although their administration is associated with some clinical side effects, and the efficiency of their ability to cross the BBB is debated.[14]

However, mAbs are not the only molecules capable of binding amyloid plaques with high specificity. There is intense scientific interest to discover small molecules which can cross the BBB and have high affinity for amyloid plaques or favor amyloid clearing or inhibit its oligo/polymerization.[14–17] Compound **Re-1** has been designed as a structural analogue of the multifaceted 2-phenylbenzothiazole pharmacophoric scaffold, a scaffold of high amyloid affinity, by replacing the phenyl moiety of 2-phenylbenzothiazole core with the cyclopentadienyl tricarbonyl [CpRe(CO)3] unit (Fig. 1A). Previous work has demonstrated effective binding

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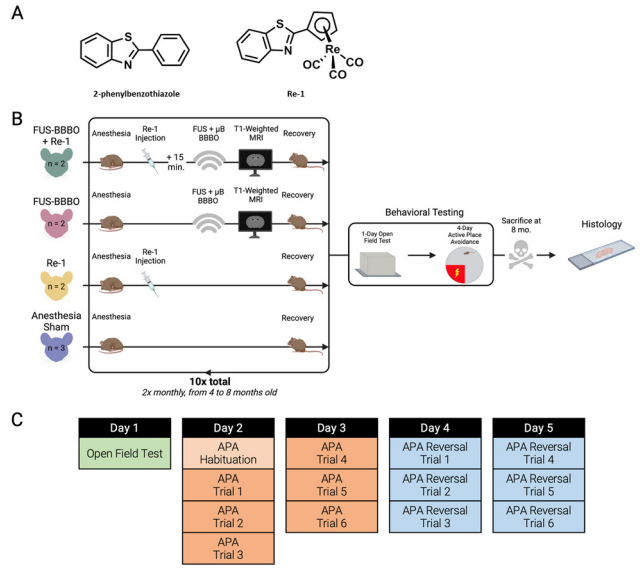

Fig. 1 Experimental overview. (A) Structures of 2-phenylbenzothiazole and **Re-1**. (B) Bi-weekly experimental protocol for each group, culminating in five days of behavioral testing, sacrifice, and immunohistological analysis. (C) Overview of behavioral testing schedule for Open Field Test, Active Place Avoidance (APA) training, and APA reversal testing.

with β-amyloid (Aβ) plaques and fibrils *in vitro*, in addition to remarkable brain penetration of this family of benzothiazolic and benzimidazolic complexes *in vivo*.[18] Further, inhibition of Aβ fibril formation and a significant reduction of Aβ-induced cytotoxicity and reactive oxygen species production in neuronal cell cultures were observed.[18]

Focused Ultrasound-induced Blood–Brain Barrier Opening (FUS-BBBO) involves the transcranial focusing of acoustic pressure waves to contact and cavitate intravenously-administered microbubbles, exerting mechanical forces on the cerebral vasculature, which gives rise to a transient increase in BBB permeability. Previous work applying FUS-BBBO alone in preclinical models has demonstrated its preventative and therapeutic efficacy for improving cognitive and pathological decline in Alzheimer's disease.[19,20] FUS-BBBO has also been used extensively to improve targeted drug and gene delivery efficiency to the brain in a noninvasive manner.[21,22] The application of FUS-BBBO with mAbs has been attempted and demonstrates moderate amyloid reduction without significant cognitive benefit.[23] Cumulatively, this foundational work motivates further investigation into the use of FUS-BBBO as a preventative therapeutic for AD, both alone and in combination with the delivery of AD therapeutics.

In this preliminary study, the efficacy of combining FUS-BBBO and **Re-1** in anxiety amelioration, memory improvement, and pathology reduction of male triple transgenic (3xTg)-AD mouse model is evaluated. Following treatment of the animals once biweekly over the course of 5 months in an early intervention paradigm for AD therapy, with FUS-BBBO only, **Re-1** only or the combination of the two, anxiety, spatial memory, and reversal learning was evaluated through behavioral testing. Furthermore, the hippocampal region of the mouse brain was used in immunohistological examination to unveil effects on amyloid fibrils or tau protein.

Materials and methods

Experimental design

This study was designed to evaluate the combined efficacy of FUS-BBBO and **Re-1** administration to the brain compared to their independent administration. This study consisted of ten, bi-weekly (once every two weeks) treatments spanning five months (Fig. 1B). Four groups (n = 2–3 per group) of four-month-old, male, triple transgenic (3xTg)-AD mice, were used. The first group received both FUS-BBBO and **Re-1**, the second received FUS-BBBO only, the third received **Re-1** only, and the final group was reserved as a sham group receiving only anesthesia at each treatment timepoint. Four months was the selected starting age of this study to begin treatment prior to the accumulation of significant pathology accumulation and cognitive deficit. In this way, this study design simulates an early intervention paradigm for AD therapy.

Focused ultrasound-induced blood–brain barrier opening and detection

Transcranial FUS-BBBO was performed as previously described.[24] Briefly, each animal was anesthetized with a mixture of oxygen and 2.0–3.0% of vaporized isoflurane and its head was fixed inside of a stereotax for FUS administration. A single-element, 1.5 MHz FUS transducer (focal length: 60 mm, diameter: 60 mm; Imasonic, France) was operated at a Peak Negative Pressure of 450 kPa with a pulse repetition frequency of 10 Hz and a pulse length of 10 000 cycles. An additional, confocally-aligned single-element transducer (V320, frequency: 7.5 MHz, focal length: 52 mm, diameter: 13 mm; Olympus NDT, Waltham, MA, USA) was used for real-time passive cavitation detection to monitor microbubble activity. In-house made microbubbles were made as previously described.[25] The bilateral hippocampus was sonicated with four distinct sonication targets. For the left hippocampus, two coordinates were targeted: (1) 1.5 mm rostral, 2.5 mm lateral and (2) 2.5 mm rostral, 1.5 mm lateral. For the right hippocampus, the same two coordinates were used: (1) 1.5 mm rostral, 2.5 mm lateral and (2) 2.5 mm rostral, 1.5 mm lateral. Baseline cavitation levels were measured at each of the four target locations, after which point a bolus injection of 8×10^8 microbubbles per μL were intravenously injected before the FUS transducer was activated at the first and third target location.

Following FUS-BBBO the animals received an IP injection of 0.2 mL Gadomide contrast agent (Omniscan, GE Healthcare, Chicago, IL, USA). Gadolinium was allowed to circulate for about 15 minutes after which the mouse was set inside of a vertical MRI (Bruker Ascend™ 400 MHz WB 9.4T) and a $T1$-weighted 2D FLASH sequence (TR: 230 ms, TE: 3.3 ms, flip angle: 70°, averages: 6, FOV: 25.6 mm × 25.6 mm, matrix size:

256 × 256, slice thickness: 0.4 mm, resolution: 0.1 mm × 0.1 mm, scan time: 5 min) was initiated to acquire MR images confirming BBB opening and hippocampal targeting. Each MRI took place roughly 30 minutes after FUS treatment.

Re-1 compound

The **Re-1** complex was prepared in moderate yields, of about 50%, from the reaction of the corresponding ferrocenyl benzothiazole derivative with the rhenium precursor $[Re(CO)_3Br_3][NEt_4]_2$ under vigorous conditions of high temperature in an autoclave container as previously described.[18] In the experimental groups that received **Re-1** compound, each animal was administered an intraperitoneal injection of the compound at a dose of 10 mg kg^{-1}, prior to FUS-BBBO in the combination therapy group.

Behavioral testing

Following the tenth and final session of therapeutic intervention, all cohorts underwent five days of behavioral testing (Fig. 1C). Behavioral testing order was randomized on each day to mitigate any confounding biases.

The Open Field Test (OFT) was performed on the first day. A 40 cm × 40 cm × 29.5 cm ($L \times W \times H$) opaque arena with an open top was used. An 8 cm-wide peripheral zone defined along the edge of the arena, and the remaining inner region (576 cm^2) was defined as a central zone. Each subject was placed in the center of the arena facing the back wall at the start of the 10-minute trial. The mouse was then allowed to explore freely while Ethovision XT tracking software (Noldus Information Technology, Leesburg, VA, USA) recorded the total distance traveled and the time elapsed in center and peripheral zones. The arena was cleaned with 70% ethanol between subject trials.

The Active Place Avoidance (APA) training and reversal trials were performed as previously described.[10] A rotating shock grid was divided into quadrants, with one designated as the shock zone. On the first day of APA training the animals were introduced to the shock grid in the absence of shock for a 10-minute habituation trial. Immediately following this trial the animals underwent the first three shock training trials, beginning each trial in the quadrant opposite the shock zone. Throughout the trial, Ethovision tracking software tracked the animal location and a 500 ms, 60 Hz, 0.5 mA foot shock was delivered upon entry into the shock zone. Additional shocks were delivered at 1.5-s intervals until the animal exited the shock zone. The performance between these same-day trials is interpreted as short-term memory and learning.

The next day, three additional APA training trials were performed in the manner described above. Comparing the performance of each animal at the end of the first day and the beginning of the second day indicates their long-term memory for the location of the shock zone.

For the final two days of behavioral testing the APA Reversal trials involved switching the quadrants designated as the 'shock' and 'opposite'. This forces the animals to learn the location of a novel shock zone and indicates their reversal learning capacity and relative levels of neural plasticity. Short- and long-term learning is defined as the performance within and between days respectively.

Tissue preparation

All animals that survived to the end of the 10 treatments, including those that were excluded from behavioral testing, were included in the subsequent immunohistochemical pathology characterization. Each animal was sacrificed by deep anesthesia and cardiac perfusion with sterile saline. The brain was dissected out, fixed in 4% PFA for 48 hours, incubated in 30% sucrose, then frozen within Optimal Cutting Temperature compound (OCT) for cryosectioning. The brains were then sectioned into 35-μm coronal sections, and Anti-Beta (β)-Amyloid antibody (AB2286) and the secondary used was Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 594) (ab150068), or Invitrogen ™ Tau Monoclonal (HT7) (Catalog No. ENMN1000) and Donkey Anti-Mouse IgG H&L (Alexa Fluor®488) as the secondary antibody the tissue was mounted on a slide and tile scan fluorescent images were acquired at 10× magnification.

Image quantification

4–5 sections were stained per subject per group and minimally processed in ImageJ to prepare for quantification. The contrast-enhanced images were then processed using a semi-automated image processing pipeline, where the hippocampi were bilaterally segmented out, thresholding and normalization was performed, and the positive fluorescent signal was quantified.

Statistical analysis & exclusion criteria

Statistical analysis for this study was performed using GraphPad Prism. All bar charts show group means. Non-parametric statistical tests were use given the small sample sizes. Kruskal–Wallis test with multiple comparisons were used to compare groups in Fig. 2A, B and 5A, B. Statistical significance is denoted as follows: * $P \leq 0.05$. Animals whose health declined and did not move during the active place avoidance shock trial were omitted from analysis (FUS-BBBO group APA trial 6 and APA reversal trial 5 & 6). Additionally, one of the

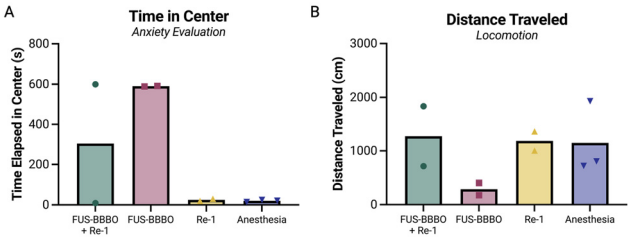

Fig. 2 FUS-BBBO + **Re-1** and FUS-only groups demonstrate reduced anxiety on the Open Field Test. (A) The average amount of time elapsed in the brightly lit OFT center. FUS-treated animals spent more time in the center than anesthesia sham animals (B) the average distance traveled over the course of the OFT trial is shown. Differences between groups are not significant by Kruskal–Wallis test with multiple comparisons for (A) and (B). n = 2–3 per group.

FUS-BBBO subjects did not exhibit normal tau pathology, and was therefore omitted from this analysis.

Results

FUS-BBBO and Re-1 may reduce anxiety

The small number of animals included in this and subsequent behavioral tests reported herein precludes making conclusive claims about behavioral performance. Rather, the results of these experiments serve as a preliminary indicator of the relative cognitive performance of each of the four groups, and the basis for future and ongoing work.

The findings presented herein demonstrate that on average, the FUS-BBBO + **Re-1** and the FUS-treated groups spent more time in the center of the arena than the **Re-1** only or Anesthesia sham groups (Fig. 2A). The amount of time elapsed in the center of the brightly-lit Open Field arena is inversely related to an animal's level of anxiety; the more time elapsed in the center, the lower the anxiety of the animal. Despite the fact that both the FUS-BBBO + **Re-1** and FUS-BBBO-only groups spent, on average, the greatest amount of time in the center out of the four groups evaluated, only the combination therapy group, FUS-BBBO + **Re-1**, did so without a drastic reduction in locomotion (Fig. 2B). Overall, this indicates that repeated FUS-BBBO may be capable of reducing anxiety as shown in prior reports,[23] but based on the average group performance, maximal benefits may result from a combination therapy with both FUS-BBBO and **Re-1**. It is important to note however, that the FUS-BBBO + **Re-1** group in particular demonstrates high inter-group variability, thus this result gives only a preliminary indication of the relative performance of each group, and further testing is necessary to conclusively establish its statistical significance.

FUS-BBBO and Re-1 may improve long-term spatial memory

The number of shocks delivered is inversely related to the spatial memory of each animal in the Active Place Avoidance (APA) paradigm. The number of shocks delivered on average to each group, per APA trial is shown in Fig. 3A. The Anesthesia sham group consistently performs the worst, receiving on average, the greatest number of shocks out of any group in five out of the six total trials. The three experimental groups performed comparably over the course of the six trials, however evaluating their performance in the context of short- and long-term memory further stratifies their performance.

The short-term memory of each group can be evaluated by comparing the number of shocks delivered to them within a single day, comparing trials 1 and 3 from the first day and 4 and 6 from the second day. Although no significant learning trend emerges in the short-term learning, the **Re-1** only group most demonstrates a reduction in the number of shocks delivered, indicating effective short-term learning on both experimental days (Fig. 3B and C).

The long-term memory of each group can be evaluated by comparing the final trial on the first day of training, trial 3, with the first trial of the second day, trial 4. The FUS-BBBO + **Re-1** group is the only group that demonstrates considerable learning over the long-term, 24-hour time scale, preliminarily indicating enhanced performance in this combination therapy group (Fig. 3D).

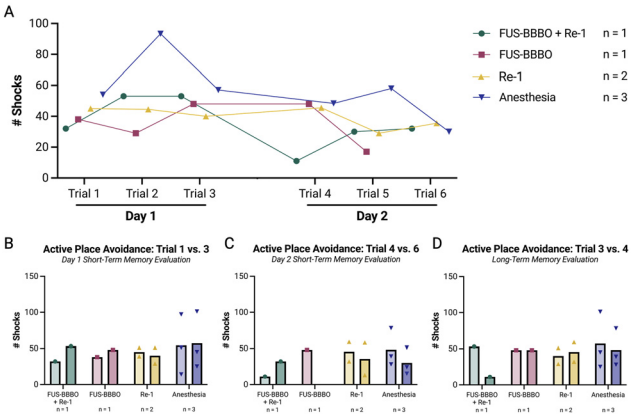

Fig. 3 FUS-BBBO + **Re-1** demonstrates the greatest long-term memory over the Active Place Avoidance training trials. (A) The average number of shocks delivered to each group over the six trials and two days of APA training is shown. The FUS-BBBO group's sixth trial is omitted as an outlier because this animal did not move over the course of the trial. The short-term memory and learning capabilities are evaluated by comparing the first and final trials for the first (B) and second (C) days of APA training. The long-term memory of each group is evaluated by comparing the final trial on the first day of training, with the first trial on the second day of training (D). n = 1–3 per group.

FUS-BBBO and Re-1 may improve short-term reversal learning

In the APA reversal trial, the number of shocks is inversely correlated to the short-term reversal learning capabilities and neural plasticity of each group. Over the course of the six APA reversal trials, the Anesthesia group again performs the worst out of all of the groups on 5 out of 6 trials (Fig. 4A). Of note, the FUS-BBBO group performance is excluded as an outlier for Trials 5 and 6, due to the fact that this animal's health declined and it did not move at all over the course of these two trials.

Interestingly, the FUS-BBBO + **Re-1** and FUS-BBBO groups demonstrated remarkable short-term reversal memory performance on the first day (Fig. 4B) and the FUS-BBBO + **Re-1** group again on the second day of reversal testing (Fig. 4C). These animals demonstrated superior neural plasticity, rapidly learning and remembering the location of a novel shock zone and demonstrating exceptional performance on the short-term for this reversal probe trial compared to the **Re-1** only and Anesthesia sham groups. Although there was improvement in both groups receiving FUS-BBBO, the FUS-BBBO + **Re-1** outperformed the FUS-only group, suggesting again that the application of this combination therapy may confer the most benefit in terms of cognitive improvement across the four experimental groups. Finally, although the **Re-1** only group did not demonstrate clear learning improvement between reversal

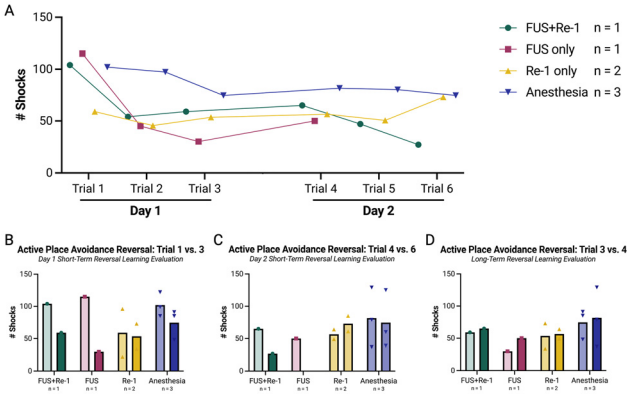

Fig. 4 FUS-BBBO + **Re-1** demonstrates the greatest short-term reversal learning in the Active Place Avoidance reversal paradigm. (A) The average number of shocks delivered to each group over the six trials and two days of APA reversal testing is shown. The FUS-BBBO group's fifth and sixth trials are omitted as outliers because this animal did not move over the course of the trial. The short-term reversal learning capacity of each group is evaluated by comparing the first and final trials for the first (B) and second (C) days of APA reversal testing. The long-term memory of each group is evaluated by comparing the final trial on the first day of training, with the first trial on the second day of training (D). n = 1–3 per group.

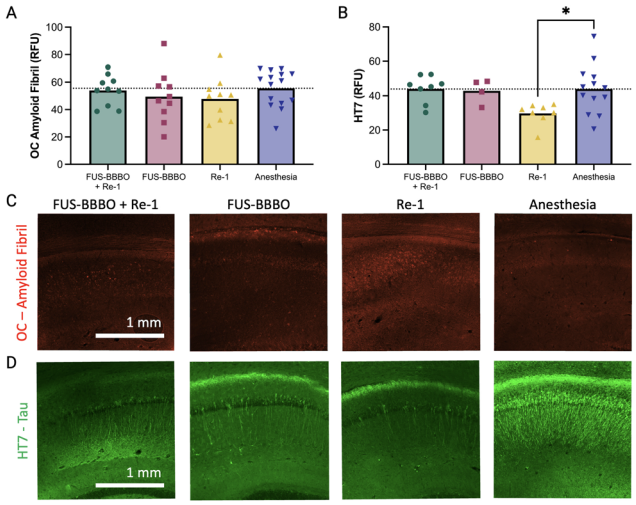

Fig. 5 **Re-1** demonstrates the greatest protection against amyloid and tau accumulation compared to anesthesia sham group. (A) The relative fluorescence values from fluorescent OC amyloid fibril-stained hippocampi are shown. The dotted line represents the anesthesia sham group average. Differences between groups are not significant by Kruskal–Wallis test with multiple comparisons. (B) The relative fluorescence values from fluorescent HT7 tau-stained hippocampi are shown. The dotted line represents the anesthesia sham group average. The **Re-1** group average is significantly lower than the anesthesia group by Kruskal–Wallis test with multiple comparisons (* $P < 0.05$). 4–5 sections were quantified per subject. Representative images from a 10× fluorescent tilescan are shown for OC (C) and HT7 (D) stained sections from each group.

trials 1 and 3, 4 and 6 or 3 and 4, they performed consistently well, receiving approximately half as many shocks as the other groups in Reversal Trial 1 (Fig. 4A).

None of the groups demonstrated considerable long-term reversal learning (Fig. 4D).

The combination of FUS-BBBO and Re-1 administration does not confer additional benefit for protecting against amyloid and tau accumulation

The data presented herein does not demonstrate significant protection against amyloid and tau accumulation with FUS-BBBO and **Re-1** combination therapy (Fig. 5). All three experimental groups (FUS-BBBO + **Re-1**, FUS-BBBO and **Re-1**) demonstrated reduced amyloid fibril accumulation on average compared to the anesthesia sham group average, indicated by the horizontal dotted line (Fig. 5A). However, differences between groups were not significant by one-way ANOVA with multiple comparisons in this preliminary study. Interestingly, tau accumulation, as measured by HT7 staining, was significantly reduced in the **Re-1** cohort compared to the anesthesia sham group ($P < 0.05$) (Fig. 5B). The reduction in tau was not significant in any other groups. Representative OC (amyloid fibril) and HT7 (tau) fluorescence images are shown in Fig. 5C and D, which qualitatively show no clear differences between the groups.

Discussion

The results presented herein provide compelling preliminary evidence for the combined efficacy of FUS-BBBO with the amyloid-binding **Re-1** compound. The known frailty of male 3xTg mice gave rise to considerable loss of animals throughout the duration of this longitudinal study and resulted in very small group sizes for final analysis and underpowered statistical tests.[26] The weights of all animals were recorded prior to each treatment and are reported in Fig. S1. All groups exhibit steady growth for the majority of the intervention period, indicating that the treatments were mostly well tolerated. However, both the Anesthesia and FUS-BBBO + **Re-1** groups average weights decline in the last month (treatments 9 and 10) when the animals were 8 months old, which may be attributed to the frailty of male 3xTg mice, which have a higher rate of mortality compared to female 3xTgs or WT mice at this age.[26] This decline in male 3xTg health and longevity indicates that perhaps the use of female 3xTg mice would be more appropriate for future longitudinal studies of this kind. Despite these small cohort sizes, we present a pilot study of the cognitive and histological outcomes from this 10-treatment early intervention paradigm. The preliminary evidence presented herein indicates a promising trend in favor of a combination therapeutic, pairing FUS-BBBO with **Re-1** delivery to the hippocampus. Notably, this study goes beyond the pathological improvement demonstrated by others with amyloid-targeting compounds, indicating that it may have the potential to improve anxiety, short- and long-term memory in the 3xTg-AD mouse model that expresses human beta amyloid and tau.

This early intervention paradigm shows the greatest efficacy in cognitive improvement with the combined FUS-BBBO and **Re-1** therapy, although FUS-BBBO also improves anxiety and reversal learning, consistent with previous reports.[20] Due to the small group sizes and underpowered statistical tests, further investigation is warranted to fully elucidate the statistical significance associated with the preliminary improvements indicated by the data presented herein. Although the combination therapy may confer additional cognitive benefit on top of the individual FUS-BBBO and **Re-1** cohorts, there was no significant benefit observed in terms of amyloid and tau accumulation. The lack of significant protection against amyloid and tau accumulation is consistent with previous reports in male 3xTg mice treated with this early intervention paradigm.[20] It is of great interest, however, that the BBB penetrating small-sized synthetic molecule, **Re-1**, significantly reduced tau accumulation, a result that is worthy of further exploration in the AD field. Larger group sizes, different dosage of **Re-1**, weekly instead of bi-weekly treatment or a longer intervention timeline enabling more extensive disease progression in the male 3xTg mice studied herein may be necessary to reveal statistically significant results. Taken together, the results of this preliminary study provide important, albeit preliminary evidence in favor of enhanced efficacy with a combination therapy of FUS-BBBO and **Re-1** for improving anxiety, long-term spatial memory and short-term reversal learning, without significant effect on amyloid and tau accumulation in 8-month-old, 3xTg, male mice.

Conclusions

The small sample size is a great limitation for this initial feasibility study the results of which, however, indicate that the combination of early intervention with FUS-BBBO and **Re-1** delivery may potentially improve anxiety, long-term spatial memory, and short-term reversal learning. This combination therapy cohort outperforms not only the anesthesia sham group, but also the independent FUS-BBBO and **Re-1**-only groups. Importantly, the data presented herein is underpowered, with too few technical replicates to make decisive claims, and thus this study is intended to serve only as a preliminary indicator of the potential of this combination therapy. The observed cognitive improvement has the potential to improve upon the majority of existing clinical AD therapeutic attempts, which achieve only pathological reduction. This study offers promising early evidence for the potential benefit of this combination therapy, warranting further investigation for AD therapy.

Institutional review board statement

All animal procedures were performed in accordance with the Guidelines for Care Use of Laboratory Animals of Columbia University and approved by the Institutional Animal Care and Use Committee under protocol #AC-AABG4559.

Author contributions

R. L. N. and E. E. K. designed the study. M. P. and M. S. provided the **Re-1** compound. R. L. N. performed FUS-BBBO and **Re-1** administration. R. L. N., A. J. B. and D. A. J. performed magnetic resonance imaging. R. L. N. and S. L. G. performed behavioral experiments. A. J. B. performed animal euthanasia. S. L. G and D. T. performed immunohistological staining and imaging. R. L. N. performed behavioral data and imaging analysis with statistical analysis. R. L. N. and E. E. K. wrote the manuscript and all authors approved its final version.

Conflicts of interest

Some of the work presented herein is supported by patents optioned to Delsona Therapeutics, Inc. where EK serves as co-founder and scientific adviser. The remaining authors declare no conflict of interest.

Data availability

Data produced for this study can be made available upon reasonable request to the corresponding author.

The average weight recorded for each group over the study duration. See DOI: https://doi.org/10.1039/d5pm00059a.

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